

Proximity to forest edge does not affect crop production despite pollen limitation

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A decline in pollination function has been linked to agriculture expansion and intensification. In northwest Argentina, pollinator visits to grapefruit, a self-compatible but pollinator-dependent crop, decline by approximately 50% at 1 km from forest edges. We evaluated whether this decrease in visitation also reduces the pollination service in this crop. We analysed the quantity and quality of pollen deposited on stigmas, and associated limitation of fruit production at increasing distances (edge: 10, 100, 500 and 1000 m) from the remnants of Yungas forest. We also examined the quantitative and qualitative efficiency of honeybees as pollen vectors. Pollen receipt and pollen tubes in styles decreased with increasing distance from forest edge; however, this decline did not affect fruit production. Supplementation of natural pollen with self- and cross-pollen revealed that both pollen quantity and quality limited fruit production. Despite pollen limitation, honeybees cannot raise fruit production because they often do not deposit sufficient high-quality pollen per visit to elicit fruit development. However, declines in visitation frequency well below seven visits during a flower's lifespan could decrease production beyond current yields. In this context, the preservation of forest remnants, which act as pollinator sources, could contribute to resilience in crop production. Like wild plants, pollen limitation of the yield among animal-pollinated crops may be common and indicative not only of pollinator scarcity, but also of poor pollination quality, whereby pollinator efficiency, rather than just abundance, can play a broader role than previously appreciated.

Keywords: Africanized honeybees; agriculture; *Citrus paradisi*; crop pollination; ecosystem services; pollen quantity and quality limitation

1. INTRODUCTION

A large proportion of the area occupied originally by many temperate and tropical terrestrial ecosystems has been converted into agricultural lands. Agriculture expansion and intensification have greatly reduced local biodiversity (Tilman *et al.* 2001), which may affect different ecosystem processes in both remaining habitat fragments and surrounding human-transformed lands. Many aspects of the functional role of biodiversity remain poorly known, but this biodiversity loss has been linked to the degradation of diverse services provided by native ecosystems, including climate regulation, pest control and crop pollination (Kremen *et al.* 2007). The loss of pollination function, in particular, is touted as a major threat to human welfare and economic yield of many crops (Allen-Wardell *et al.* 1998; Steffan-Dewenter *et al.* 2005; Klein *et al.* 2007). Indeed, 30% of the food supply by volume consumed directly or indirectly by humans depends on animal pollination (McGregor 1976), with bees being the most important pollen vectors (Roubik 1995; Klein *et al.* 2007). Thus, pollination is often characterized as a crucial ecosystem

service in decline, although this issue has been the subject of much debate in scientific and policy circles as well as among the general public (Ghazoul 2005).

How vulnerable are different crops to pollinator declines? A recent review reported that production of 37 out of the 57 globally most important crops increases with pollinating animals, stressing the importance of plant–pollinator interactions for modern agriculture (Klein *et al.* 2007). However, beyond these raw estimates, the basic pollination requirements of many crops are unknown and the contribution of wild bee communities to fruit and seed production for the majority of crops is unclear. Thus, the study of the relative importance of managed and unmanaged pollinators in crop pollination provides both essential information for food production and the conservation and management of landscape mosaics. Here we assess the importance of remnants of a subtropical forest in providing a pollination service for neighbouring crops by studying whether, and how distance to the forest edge influences pollination and fruit set in grapefruit. Despite a few studies which assessed crop pollination in relation to proximity to remnants of natural or semi-natural habitats (revised in Kremen *et al.* 2007), this is the first to explore mechanistically how an inadequate pollination might limit crop production and the quantitative and qualitative role of pollinator efficiency in causing this limitation.

The species richness and abundance of crop pollinators often decline with distance from natural or semi-natural

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Electronic supplementary material is available at <http://dx.doi.org/10.1098/rspb.2007.1547> or via <http://journals.royalsociety.org>.

habitats in North (Kremen *et al.* 2004; Morandin & Winston 2005), Central (Ricketts 2004) and South America (Chacoff & Aizen 2006), Asia (Klein *et al.* 2003), Europe (Steffan-Dewenter *et al.* 2002) and Australia (Blanche *et al.* 2006). However, the consequences of these declines for crop productivity have received much less attention (Ghazoul 2005). Among the few available studies, Kremen *et al.* (2002) found that high bee abundance and diversity were essential for pollen deposition in watermelon and varied positively with proximity to natural habitat. In addition, Ricketts (2004) demonstrated that coffee flowers on plants within 100 m of the nearest forest fragment in Costa Rica received more pollen than those on more distant plants. Unfortunately, the processes responsible for these negative associations have not been determined.

Declines in visit frequency, or average pollinator efficiency associated with an impoverished pollinator community, can reduce both the quantity and quality of the pollen deposited on stigmas, which in turn can cause pollen limitation for fruit and/or seed production (Knight *et al.* 2005). The pollen quantity may be limiting if pollinators are scarce, if they contact floral sexual organs infrequently, or if plants compete for the services of shared pollinators (Bierzychudek 1981). Even with an adequate pollinator supply, pollen quality may limit plant fecundity if, for instance, pollinators deliver mostly self-pollen or pollen from close relatives (Waser & Price 1991).

In a recent review considering 482 studies of the effect of pollen supplementation on fruit set in wild plants, Knight *et al.* (2005) found that 63% of species exhibited pollen limitation in at least some sites or years. Although sample sizes for cultivated plants are more limited, Thomson & Goodell (2001) found evidence of pollen limitation among 59% of 16 crop species. These figures suggest that pollen limitation is common, so that any pollinator decline associated with habitat destruction could further compromise the reproduction of many wild and cultivated plants.

Grapefruit (*Citrus paradisi* Macf.) is a widely cultivated crop in the Andean piedmont of northwest Argentina, a region formerly occupied by a species-rich, transitional, subtropical forest known as the lowland Yungas. In this region, grapefruit flowers are pollinated mostly (greater than 90% of flower visits) by wild Africanized honeybees. Despite the alien origin of this bee, we found that visitation frequency by honeybees decreased by greater than 50% from the forest edge to 1 km inside plantations during the 3 year period. The few recorded visits by native bees occurred mostly within 100 m of forest remnants (Chacoff & Aizen 2006). In addition, hand- and natural-pollinated flowers resulted in about six times more fruit than emasculated and bagged (insect-excluded) flowers and cross-pollen performed better than self-pollen, in terms of germination and tube growth (Chacoff & Aizen 2007). Thus, these grapefruit plantations provide the opportunity to examine the effects of isolation from natural forest on pollination and fecundity.

In this study, we evaluated whether the observed decrease in pollinator visitation with increasing distance to forest edge additionally reduced pollination service in grapefruit plantations and the processes responsible for variation in fruit set. In particular, we assessed (i) whether pollination success, estimated as both pollen grains on the stigmas and pollen tubes in styles, declined with distance from forest edge and (ii) whether fruit set decreased, and

variability in fruit production increased with distance from the forest edge. In addition, we used a pollen-supplementation experiment, in which we varied both pollen amount and origin (self versus cross) to determine (iii) whether pollen quantity or quality limited fruit set in grapefruit and (iv) whether the severity of pollen limitation increased with distance from the forest edge. Finally, we assessed quantitative and qualitative aspects of the pollination efficiency of wild Africanized honeybees to study (v) whether pollen deposition increased with successive honeybee visits and the performance of the pollen they deposited compared to pure self- and cross-pollination standards. In addition to describing the patterns of plant pollination and reproduction associated with a decline in pollinator visitation (i and ii), the mechanistic insights exposed by this study (iii–v) allow us to address alternative explanations for the patterns observed. This detailed knowledge of reproductive processes provides a sound basis for predicting the consequences of a pollinator decline and for identifying effective options for managing the yield of animal-pollinated crops.

2. MATERIAL AND METHODS

(a) Study area, species and experimental design

We conducted this study in the Upper Bermejo River Basin, Salta province, northwest Argentina. The landscape in this region is dominated by degraded forest remnants neighbouring an expanding agriculture matrix of extensive plantations of citrus, sugarcane and soya bean, and smaller plantations of avocado and mango. Biogeographically, these forest remnants correspond to the premontane forest of the Yungas, a narrow strip of subtropical, semi-deciduous forest that extends, in Argentina, along discontinuous mountain ranges between 22 and 29° S. With an annual deforestation rate of 2.3% (Brown & Malizia 2004), this lowland forest represents one of the most rapidly transformed biomes in southern South America. Annual rainfall in this region averages 733 mm and varies between 280 and 1224 mm (Brown *et al.* 2001).

Grapefruit planted in the study area belongs mostly to red and very red seedless varieties. Despite self-compatibility and partial parthenocarpy, the study varieties are highly dependent on insect pollination for fruit production (Chacoff & Aizen 2007, more details in the electronic supplementary material). We selected four extensive, single-variety grapefruit plantations, more than 1 km wide, including La Toma (23°21'44" S, 64°18'18" W), Citrusalta (23°28'15" S, 64°24'20" W), Manero (23°25'33" S, 64°26'30" W) and Peña Colorada (22°47'58" S, 64°22'13" W). Each plantation bordered relatively continuous forest along one side and was surrounded by other citrus plantations or other crops (e.g. soya bean) at the other sides. Minimum distance between plantations (approx. 5 km) exceeds the typical foraging ranges of most local bees (Roubik 1995). Plantations were managed using similar weed and pest control protocols, owing to certification requirements for fruit export.

At each plantation, we considered five distances from the forest edge: 0 m or 'edge' (the first row of grapefruit trees adjacent to the forest edge), 10 m (the third row), 100, 500 and 1000 m. At the beginning of the 2000 flowering season, we selected 20 focal plants randomly at each distance in each of the four plantations to assess pollination and fruit set during three consecutive flowering seasons (2000–2002) and their corresponding fruiting seasons (2001–2003).

(b) Pollination and fruit set

To assess the pollination service provided by pollinators from nearby semi-natural habitat remnants to the four grapefruit plantations, we estimated stigmatic pollen receipt and performance of pollen tubes in the style as well as fruit set at increasing distances from the forest edge. We also assessed whether the amount and origin of the pollen deposited on stigmas limited fruit set with a pollen-supplementation experiment.

At the beginning of the flowering season, we tagged nine branches on each of 20 focal grapefruit plants per site. After counting flowers and flower buds, we assigned each branch randomly to one of three treatments (i.e. three branches per treatment per focal plant): (i) natural pollination (i.e. flowers exposed to pollinators), (ii) supplementation with self-pollen, and (iii) supplementation with cross-pollen. Pollen supplementation (treatments 2 and 3) was achieved by rubbing fresh anthers of five flowers from the same plant (self) or from five different grapefruit trees (cross) on receptive stigmas of flowers otherwise exposed to natural pollination. Flowers were hand-pollinated at least once during their lifespan (see electronic supplementary material for more details).

During the 2001 and 2002 flowering seasons, we collected styles approximately one week after flower senescence, just before they started falling naturally; to count pollen grains on the stigmas and pollen tubes in the styles of both natural- and hand-pollinated flowers. We collected styles in order to evaluate stigmatic pollen receipt and performance of pollen tubes in the style. Styles were fixed and stored in individual microcentrifuge tubes containing FAA (formalin : acetic acid : ethyl alcohol, 5 : 5 : 90). In the laboratory, styles were cleared in a 10 ml l^{-1} NaOH solution for 24 hours and stained with 0.1% aniline blue in 0.1 mol l^{-1} K_3PO_4 . Squashed preparations were examined with an epifluorescence microscope at $100\times$. For each style, we counted the number of pollen grains on the stigma and the number of pollen tubes at the base of the style. In total, we collected 1023 styles, averaging (\pm s.d.) 70 ± 9 styles per pollination treatment and distance class.

During March, after each study flowering season, we counted ripe fruits from tagged branches. We determined fruit set (i.e. number of fruits/number of flowers) for each sampled distance from the forest edge and pollination treatment from a total of 37 637 flowers.

(c) Honeybee efficiency

We evaluated the quantitative and qualitative efficiency of *A. mellifera* as a pollinator of grapefruit flowers. During the 2003 flowering season, we selected eight trees at La Toma plantation. Several branches were bagged with nylon mesh to prevent insect visits. Grapefruit pollen is not wind-borne, but bagging does not prevent autonomous, within-flower self-pollination (Chacoff & Aizen 2007). As they opened, flowers were marked with jewellery tags and exposed to free-foraging pollinators. We counted the number of legitimate visits received by each flower during differing periods, identifying a legitimate visit when an insect contacted the stigma and/or anthers. Some flowers were rebagged and exposed to pollinators more than once, even during two consecutive days. We monitored a total of 190 flowers receiving between 0 and 9 visits. We recorded only 2 out of 222 visits by insects other than *A. mellifera*; so we restricted our analysis of pollination efficiency to honeybees. As described in Chacoff & Aizen (2007), we also hand-pollinated

847 and 500 virgin flowers once during their lives with either self- or cross-pollen, respectively. These flowers were rebagged after pollination and thus were not exposed to pollinators. Styles of natural- and hand-pollinated flowers were collected, treated and observed as described above.

(d) Statistical analysis

We analysed the effects of distance to the forest edge and pollen supplementation on the numbers of pollen grains on stigmas, pollen tubes at the base of styles, fruit set (number of fruits/number of flowers) and the coefficient of variation (CV) of fruit set among branches, using generalized linear-mixed models (GLIMMIX procedure of SAS; SAS 2004). We contrasted two models to describe the relation of pollen receipt (y) to the number of honeybee visits to flowers (x). We adjusted a linear equation, $y = y_0 + bx$ and a saturating negative exponential model $y = y_0 + a(1 - \exp^{-bx})$, where y_0 is the number of pollen grains without visitation, which accounts for some autonomous, self-pollen deposition. See extended details of the statistical analysis and selection of models in the electronic supplementary material.

3. RESULTS**(a) Natural pollination and fruit set**

Overall, stigmas of grapefruit flowers had an average (\pm s.e.) of 59.8 ± 4.12 germinating pollen grains. Owing to attrition in the style, an average of only 9.3 ± 2.23 pollen tubes reached the base of the style.

For natural-pollinated flowers, the numbers of both pollen grains on the stigma and pollen tubes in the style varied negatively with distance to the forest edge (electronic supplementary material, table 2). Stigmatic pollen loads decreased linearly from the forest edge into the plantation ($y = 65.07 - 0.015x$, electronic supplementary material table 2, solid line in figure 1a), so that flowers on trees at 1000 m received 22.5% fewer pollen grains than those adjacent to the forest edge. Pollen tubes in the style followed a similar trend ($y = 10.14 - 0.003x$, electronic supplementary material table 2, solid line in figure 1b) with an approximate 30% difference in the number of pollen tubes between grapefruit plants at less than 100 and 1000 m from the forest edge. Out of 24 066 natural-pollinated flowers, only 834 developed into fruit (i.e. 3.5%). Neither the mean fruit set nor its variability varied significantly with distance to forest edge (electronic supplementary material table 2, solid line in figure 1c,d).

(b) Pollination and fruit set following pollen supplementation

Pollen supplementation increased pollination and fruit set, while decreasing variability in fruit production, regardless of whether supplementation involved self- or cross-pollen (electronic supplementary material, table 3; figure 1). Supplemented stigmas (cross- and self-pollinated treatments pooled) had, on average, 49.5% more pollen grains, which translated into 32.7% more pollen tubes in the style than natural-pollinated flowers. Although the number of pollen grains on stigmas did not differ statistically between flowers supplemented with self- versus cross-pollen, the number of pollen tubes reaching the base of the style was approximately 10% greater for flowers supplemented with cross-pollen (electronic supplementary material, table 3). Supplemented flowers

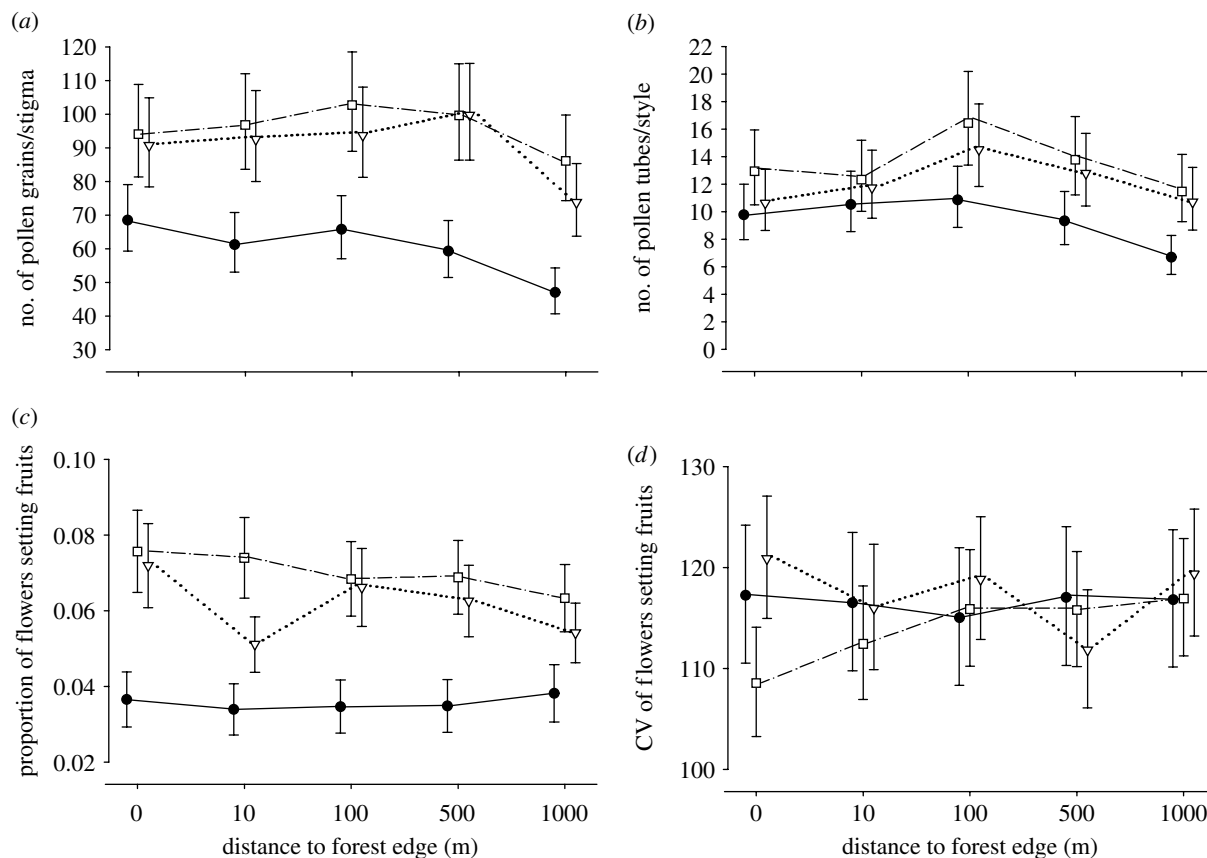


Figure 1. Least-squares mean (\pm s.e.) pollination success. (a) Number of pollen grains on the stigma, (b) number of pollen tubes in the style, (c) fruit set and (d) CV in fruit set for grapefruit (*C. paradisi*) at different distances from the forest edge. (Circles, natural pollination; squares, cross-pollination; inverted triangles, self-pollination.)

set 36.2% more fruit than natural-pollinated flowers, and those supplemented with cross-pollen set approximately 18% more fruit than those supplemented with self-pollen. Supplemental pollination reduced variability in fruit set by 14.7%, with marginally less variability following supplementation with cross-pollen than with self-pollen (electronic supplementary material, table 3). Unlike natural-pollinated flowers, distance from the forest edge did not affect stigmatic pollen loads, numbers of pollen tubes or mean and variability in fruit set for flowers subject to supplemental pollination (electronic supplementary material table 2, figure 1).

The random factors, year and site, and some of their interactions with the fixed factors remained in the final models indicating some spatio-temporal variability among years and plantations in the pollination and reproductive response variables. However, these sources of variation were smaller than the variability among trees within plantations (electronic supplementary material, table 4; figure 1), an unexpected result, given the clonal origin of these plantations.

(c) Honeybee efficiency

Despite considerable variation, pollen deposition by honeybees tended to increase with number of honeybee visits. A saturating negative exponential function provided a better fit to this relation than a linear model (figure 2). Whereas unvisited flowers had, on average, 14.1 pollen grains; the asymptotic mean pollen load was 54.9 grains (95% CI=47.1–67.5 grains). Seven bee visits were

sufficient to deliver 90% of this asymptotic mean. Although few flowers in this sample experience more than seven visits, there was no indication that additional visits significantly increased pollen delivery (figure 2). Pollen delivered by honeybees produced fewer pollen tubes at the base of the style than hand-delivered self- or cross-pollen, as measured by the asymptotic mean number of pollen tubes (figure 3).

4. DISCUSSION

Remnants of natural or semi-natural habitat can act as a source of pollinators for nearby crops (Kremen *et al.* 2007). In the study plantations, we observed few visits to grapefruit flowers by native bees far from the forest edge, whereas the abundance of the omnipresent, Africanized honeybee also declined by half within 1 km from the forest (Chacoff & Aizen 2006). Such effects of habitat edge and fragmentation on pollinator diversity and abundance have raised concerns of a more global decline in pollinator abundance (Steffan-Dewenter *et al.* 2005); however, few studies have considered the associated consequences for crop pollination (Kremen *et al.* 2002; Klein *et al.* 2003; Morandin & Winston 2005; Greenleaf & Kremen 2006).

Our study of grapefruit pollination demonstrated that a twofold decrease in visitation frequency by honeybees with distance from forest edge caused a 20–30% decrease in pollination. Current rates of honeybee visitation ensure that even at 1 km from the forest grapefruit flowers may receive 15 or more visits, on average, over their lifespan

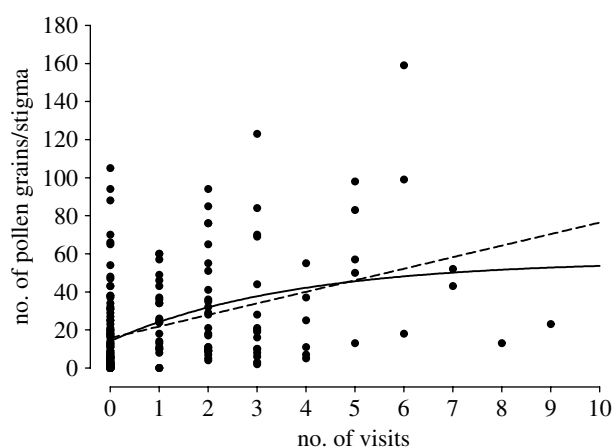


Figure 2. Relation of the number of pollen grains deposited on the stigmas to the number of honeybee visits to individual grapefruit flowers ($N=190$). The fitted curves are (solid) $y=14.08+40.79(1-\exp^{-0.29x})$ and (dotted) $y=14.86+6.84x$. The saturating function provides a better fit ($AIC=5367$) than the linear function ($AIC=5421$).

(Chacoff & Aizen 2006). Whereas this visitation frequency must suffice to reach asymptotic pollination (figure 2), high variability in visitation rates may increase the proportion of flowers receiving less than seven visits far from the forest edge. In addition, we observed that native bees (e.g. *Geotrigona argentina* Camargo and Moure, *Tetragonisca angustula* Latierre, *Bombus atratus* Franklin, *Augochloropsis cupreola* Cockerell) account for approximately 15% of the visits to grapefruit flowers near the forest edge, a proportion that becomes zero at 1 km from the edge (Chacoff & Aizen 2006). Although we did not measure the pollination efficiency of these bees, they might be responsible for the 30–40% increase in pollination, observed near forest edges, over honeybee maximum efficiency (figures 1 and 2). This result opens the intriguing possibility that promoting the abundance of alternative pollinators may improve grapefruit pollination.

The decline in pollination with increasing distance from forest was not accompanied by a parallel decline in grapefruit production, despite clear evidence of pollen limitation. Quantitative and qualitative influences on fruit set may explain this discrepancy.

First, the relation of fruit production to pollination can be clearly nonlinear (Harder & Thomson 1989), so that pollen receipt may be a poor indicator of fruit set potential. As reported for cranberry (Cane & Schiffhauer 2003), pollen receipt must exceed a minimum threshold to elicit fruit set (Brown & McNeil 2006; Aizen & Harder 2007). In addition, the ‘mass effect’ produced by the simultaneous deposition of pollen on the stigma could perhaps be more important than total pollen deposition (Ganeshaiah & Shaanker 1988). Despite the partial parthenocarpic nature of the varieties that we studied, the stimulus provided by pollen grain deposition and germination seems critical for tube growth and fruit development (Chacoff & Aizen 2007). Thus, the intensity of this stimulus could depend more on the number of pollen grains deposited during a single visit than on the number of grains accumulated during a flower’s lifespan. This mass effect might explain why deposition of a given number of pollen grains during several honeybee visits resulted in lower fruit set than a single deposition of the

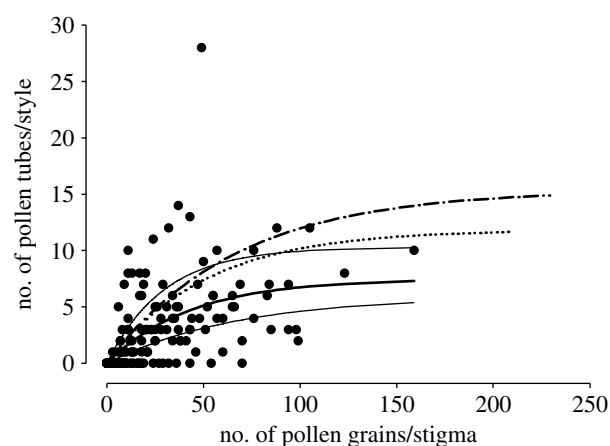


Figure 3. Saturating negative exponential curves of the form $y=a(1-\exp^{-bx})$, describing the relation of the number of pollen tubes reaching the base of the style to stigmatic pollen load for grapefruit flowers exposed to honeybees ($N=190$ flowers) and flowers that were hand-pollinated with either pure self- and cross-pollen ($N=111$ and 110 , respectively). Parameter estimates were $a=7.49$ (95% CI=5.98–10.29) and $b=0.023$ (0.015–0.034) for honeybee pollination, 11.9 (10.5–13.9) and 0.019 (0.014–0.025) for self-pollination and 15.4 (13.3–18.5), 0.015 (0.010–0.020) for cross-pollination. For clarity, the 95% CI and observations are shown for honeybee pollination only (solid line, *A. mellifera*; dashed-dotted line, cross-pollen; dotted line, self-pollen).

same amount of either self- or cross-pollen (figure 3). Similarly, this mass effect may explain the large response in fruit production caused by pollen supplementation (figure 1c). In any event, honeybees may not deposit enough pollen during either single visits or flower lifespan to maximize grapefruit production.

Second, pollen transferred to the stigmas by honeybees could also be of genetically poor quality. Despite being able to fly long distances, individual honeybees concentrate on their foraging on spatially restricted nectar and pollen sources, usually a small flowering patch or the canopy of a single tree (Javorek *et al.* 2002). In many cases, this behaviour results in exclusive self-pollination. Approximately 90% of 1931 consecutive movements that we observed in honeybees visiting grapefruit were between flowers of the same tree, indicating that the behaviour needed for cross-pollination may occur infrequently (N. Chacoff 2002, unpublished data). Differences in pollen performance and fruit production after supplementation with self- versus cross-pollen in our study suggest that pollen quality can be an important determinant of grapefruit yield. More generally, limitation of fruit and seed production by poor-quality pollination may be more common in both wild and cultivated plants than previously appreciated (Aizen & Harder 2007). Thus, the maintenance of genetic variation within plantations can be an important, but underestimated, factor in crop production.

5. CONCLUDING REMARKS

The more mechanistic approach we have followed to study edge effects on crop pollination, including recognition of pollen quality as an essential factor affecting plant reproductive dynamics, led us to a deeper and broader understanding of the potential consequences of pollinator

decline. Our results show that pollinator efficiency can play a broader role than previously appreciated, providing circumstantial evidence of why reductions in pollinator diversity can be more detrimental for the pollination function than just decreases in pollinator abundance (Klein *et al.* 2003). By assessing different components of honeybee efficiency as a grapefruit pollinator, we were able to address the causes of pollen limitation in crop yield. At the same time, we advanced our general understanding of the mechanisms leading to this type of reproductive limitation in flowering plants (Aizen & Harder 2007). This knowledge has clear implications for management of grapefruit production in northwest Argentina that may also apply to other crops elsewhere. First, introduction of honeybee hives into grapefruit plantations is unlikely to increase fruit production above the pollination service provided for free by the environment, because this bee's pollination efficiency reaches a ceiling after a few number of visits. However, declines in visitation frequency well below seven visits during a flower's lifespan could decrease grapefruit production beyond current yields. In this context, the preservation of forest remnants, which act as pollinator sources, could contribute to resilience in crop production. Second, despite being the dominant flower visitor in grapefruit plantations, *A. mellifera* is not necessarily a quantitatively efficient pollinator of this crop and other plant species (e.g. Adler & Irwin 2006). In addition, the pollen delivered by honeybees was genetically and/or physiologically poor that it caused quality limitation. At present, agricultural practice often ignores whether native bees might be more efficient pollinators than honeybees, or whether the promotion of these alternative pollinators is feasible and economically viable. However, the observed increase in grapefruit pollination near the forest edge, where these pollinators were relatively more abundant, suggests interesting management perspectives in this respect. Finally, given that pollen quality could be as important as pollen quantity in determining grapefruit production, genetic variation, even within single-variety plantations, should be preserved and promoted. Thus, our study shows that whereas remnants of natural or semi-natural habitats can provide pollinators and pollination to neighbouring plantations, this ecosystem service, which is worth preserving, may not be enough to overcome pollen limitation. However, an understanding of the quantitative and qualitative mechanisms leading to this limitation can prove crucial for improving yield of many animal-pollinated crops.

The authors would like to thank A. Klein, S. Cunningham, L. Harder and D. Vázquez for their helpful comments and suggestions on the earlier versions of this manuscript. R. Lobo, C. Monmany and G. Elias provided unconditional assistance during fieldwork. J. Morales provided much valuable assistance for fitting models to data. A. Brown provided logistic assistance during this work. The authors are also grateful to R. Manero, D. Lorenzo, J. Campos and R. Burgos for allowing them to conduct this study in their plantations. The Antorchas Foundation (grant 14022-22), ProYungas Foundation, Sigma Xi and the National Research Council of Argentina (CONICET) funded this research. The first and third authors hold a postdoctoral and doctoral fellowship, respectively and the second author holds a researcher position from CONICET.

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